



## AIR FORCE RESEARCH LABORATORY

### Identification of Gene Expression Changes in Whole Blood Indicative of Exposure to Chemicals with Different Target Organ Toxicity

Victor Chan  
Andrea Stapleton  
**Alion Science and Technology Corp.**

Armando Soto  
Kyung Yu  
Nicholas DelRaso  
**Human Effectiveness Directorate**

**February 2006**

**20061128063**

Approved for public release;  
Distribution is unlimited.

Air Force Research Laboratory  
Human Effectiveness Directorate  
Warfighter Interface Division  
Cognitive Systems Branch  
Wright-Patterson AFB OH 45433

# REPORT DOCUMENTATION PAGE

*Form Approved  
OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

|  |                    |                     |  |                     |   |  |
|--|--------------------|---------------------|--|---------------------|---|--|
| 1. REPORT DATE (DD-MM-YYYY)  |                    |                     | 2. REPORT TYPE   |                     | 3. DATES COVERED (From - To)                                      |  |
| 4. TITLE AND SUBTITLE  |                    |                     | Chasing the Sun -The In-flight Evaluation of an Optical Head Tracker |                     | 5a. CONTRACT NUMBER<br>F33615-00-C-6060                           |  |
|  |                    |                     |  |                     | 5b. GRANT NUMBER  |  |
|  |                    |                     |  |                     | 5c. PROGRAM ELEMENT NUMBER  |  |
| 6. AUTHOR(S)   |                    |                     |  |                     | 5d. PROJECT NUMBER<br>7184  |  |
| Victor Chan *  |                    |                     |  |                     | 5e. TASK NUMBER<br>D4   |  |
| Andrea Stapleton *   |                    |                     |  |                     | 5f. WORK UNIT NUMBER<br>05  |  |
| Armando Soto **  |                    |                     |  |                     |   |  |
| Kyung Yu **  |                    |                     |  |                     |   |  |
| Nicholas DelRaso **  |                    |                     |  |                     |   |  |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)<br>AND ADDRESS(ES)  |                    |                     |  |                     | 8. PERFORMING ORGANIZATION REPORT NUMBER                          |  |
| * Alion Science and Technology Corp., Dayton OH<br>** Human Effectiveness Directorate, Wright-Patterson AFB OH   |                    |                     |  |                     |   |  |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)  |                    |                     |  |                     | 10. SPONSOR/MONITOR'S ACRONYM(S)<br>AFRL/HEPB                     |  |
| Air Force Materiel Command<br>Air Force Research Laboratory<br>Human Effectiveness Directorate<br>Biosciences and Protection Division<br>Applied Biotechnology Branch<br>Wright-Patterson AFB OH 45433 |                    |                     |  |                     | 11. SPONSOR/MONITOR'S REPORT NUMBER(S)<br>AFRL-HE-WP-TP-2006-0032 |  |
| 12. DISTRIBUTION / AVAILABILITY STATEMENT<br>Approved for public release; distribution is unlimited. Cleared by AFRL/WS-06-0424 on 15 February 2006.   |                    |                     |  |                     |   |  |
| 13. SUPPLEMENTARY NOTES  |                    |                     |  |                     |   |  |
| 14. ABSTRACT   |                    |                     |  |                     |   |  |
| 15. SUBJECT TERMS<br>Azimuth Measurement, Head Tracker, Helmet-Mounted Trackers, Dynamic Measurement, Static Measurement   |                    |                     |  |                     |   |  |
| 16. SECURITY CLASSIFICATION OF:  |                    |                     | 17. LIMITATION OF ABSTRACT   | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON<br>Diane Todd                     |  |
| a. REPORT<br>UNC   | b. ABSTRACT<br>UNC | c. THIS PAGE<br>UNC | SAR  | 17                  | 19b. TELEPHONE NUMBER (include area code)                         |  |

Standard Form 298 (Rev. 8-98)  
Prescribed by ANSI Std. Z39-18

## IDENTIFICATION OF GENE EXPRESSION CHANGES IN WHOLE BLOOD INDICATIVE OF EXPOSURE TO CHEMICALS WITH DIFFERENT TARGET ORGAN TOXICITY

Victor Chan<sup>1</sup>, Andrea Stapleton<sup>1</sup>, Armando Soto<sup>2</sup>, Kyung Yu<sup>2</sup>, and Nicholas DelRaso<sup>2</sup>

<sup>1</sup>Alion Science and Technology, Corp., and <sup>2</sup>Applied Biotechnology Branch, Division of Biosciences and Protection, Human Effectiveness Directorate, Air Force Research Laboratory, Wright-Patterson AFB, Dayton, Ohio.

### ABSTRACT

Toxicogenomic profiling is a molecular approach to capture the global, transcriptomic response of a biological system caused by perturbations resulted from chemical exposure. Coupled with advanced bioinformatic techniques, it allows for the elucidation of the molecular mechanisms of chemical toxicity, as well as the identification of novel biomarkers predictive for chemical exposure. In an effort to investigate if gene expression changes in whole blood can serve as novel biomarkers indicative of organ-specific malfunction resulted from chemical exposure, we have initiated a transcriptomic profiling study using chemicals with different target organ toxicity in a rat model. The first three compounds chosen were  $\alpha$ -naphthylisothiocyanate (ANIT, 0.1 – 100 mg/kg), chlorpyrifos (CPF, 0.5 – 50 mg/kg) and 2-bromoethylamine (BEA, 1.0 – 250 mg/kg), which are model hepatotoxin, neurotoxin and nephrotoxin, respectively. Gene expression profiles of blood samples isolated from control and chemical-treated rats were determined using DNA microarrays (Affymetrix GeneChip, RAE230A). The expression levels of 584, 303 and 441 genes were significantly altered after chemical treatments as determined by t-test, ANOVA and SAM (significance analysis of microarray), respectively. Close examination of these gene lists revealed that there are a total of 1075 unique genes that collectively comprised these lists. Of these unique genes, 213 were identified by two methods, while 40 genes were common in all three methods. Using a dataset of the expression profiles of these 40 genes as input data in hierarchical clustering and principal component analysis demonstrated that these are useful discriminators for separating animal groups treated with different chemicals. This result strongly supports the premise that whole blood is a suitable surrogate tissue for monitoring gene expression changes for detection of organ-specific toxicity. Future efforts will involve transcriptomic profiling of additional chemicals, and validate gene expression changes identified in this experimental model as biomarkers for specific exposure paradigms and real-world exposure scenarios.

### OBJECTIVES

1. To investigate whole blood gene expression changes in rats exposed to  $\alpha$ -naphthylisothiocyanate, chlorpyrifos and 2-bromoethylamine, the model hepatotoxin, neurotoxin and nephrotoxin, respectively.
2. To compare gene expression changes in rats exposed to chemicals with different target organ toxicity.

3. To investigate if whole blood gene expression changes in rats can be used as surrogate biomarkers in rats exposed to chemicals with different target organ toxicity.

## METHODS AND MATERIALS

### 1. Animals, Chemical Treatment and RNA Extraction

- a. Male Fischer F344 rats, 222-258g, were obtained from Charles River Laboratories.
- b. Animals were dosed by oral gavage with chemicals or vehicle alone:
  - ANIT in corn oil: 0.1, 0.5, 1.0, 10, 20, 50 & 100 mg/kg
  - CPF in corn oil: 0.5, 1.0, 10, 30 & 50 mg/kg
  - BEA in normal saline: 1.0, 5.0, 15, 50, 150 & 250 mg/kg
- c. Control and chemical-treated animals was sacrificed by CO<sub>2</sub> inhalation at 96 hours post dose
- d. 1.5-2.5 ml blood from each animal was collected into a syringe and immediately added into each PAXgene tube. The sample was immediately inverted several times to ensure complete mixing with the reagents. After incubation at room temperature overnight to allow for lysis of the blood cells, the samples were stored at -80°C until further processing. The extraction of total RNA from the processed blood samples was performed as recommended by the manufacturer.

### 2. Gene Expression Profiling and Fingerprinting

- a. Affymetrix oligonucleotide GeneChip arrays (RAE230A) were used for mRNA expression (transcriptomics) profiling according to the Affymetrix GeneChip Expression Analysis Technical Manual
- b. Total RNA was isolated from tissues isolated from treated or untreated animals using Qiagen RNeasy Mini kit (Qiagen, Valencia, CA).
- c. Fifteen µg of total RNA was used for first strand cDNA synthesis using the SuperScript Choice system (Invitrogen Corporation, Carlsbad, CA) in the presence of an oligo-(dT)24 anchored T7 primer (Proligo, Boulder, CO) followed by 2nd strand synthesis.
- d. Biotinylated RNA was synthesized using BioArray high yield RNA transcript labeling kit (Enzo Diagnostics, Inc., Farmingdale, NY) and fifteen µg of fragmented bio-cRNA was then hybridized with Affymetrix Rat Expression 230A gene array.
- e. Hybridized and stained chips were scanned with Affymetrix GeneChip Scanner 3000 and the intensity was captured using Affymetrix GeneChip Operating Software 1.1 according to Affymetrix standard procedures.
- f. A single expression level for each gene was derived from the 11 probe pairs representing each gene; 11 perfectly matched (PM) and 11 mismatched (MM) control probes. The MM probes act as specificity controls that allow for the direct subtraction of background cross-hybridization signals.

### 3. Data Filtering, Statistical Analysis and Visualization of Analysis Results

- a. The gene expression status and/or signal quality were determined using the Detection Algorithm of the Affymetrix Data Mining Tool (DMT v. 3.1). Genes received "Absent" call across all samples in the experiment of one chemical were considered as "uninformative".

- Genes with uninformative status in two or more experiments will be removed from the dataset.
- b. Gene expression changes were identified using three different statistical methods: t-test, ANOVA and SAM using the Affymetrix DMT and TIGR TMeV.
  - c. T-test: Gene expression changes were determined for each chemical.
    - Only genes with  $p < 0.01$  in two consecutive doses are considered significant.
    - All the significant genes were then combined as significant gene expression changes.
  - d. ANOVA: Gene expression changes were determined for each chemical.
    - Only genes with  $p < 0.01$  are considered significant.
    - p-value was determined based on 1000 permutations.
    - False discovery rate for multiple measurements was controlled by adjusted Bonferroni procedure [8, 9].
  - e. SAM: Gene expression changes were determined using multi-class analysis procedure.
    - Four classes were assigned to the samples.
    - Control: Control samples from all experiments were pooled.
    - ANIT: All ANIT samples assigned to this class regardless the doses.
    - CPF: All CPF samples assigned to this class regardless the doses.
    - EBA: All BEA samples assigned to this class regardless the doses.
  - f. Classification of samples using different datasets as input data by Principal Component Analysis (PCA) was performed using Partek Pro.

## RESULTS

1. 6113, 6299 and 5778 genes were identified as “informative” in the ANIT, CPF and BEA datasets, respectively. Of these genes, 5426 genes were informative in all three datasets, while 599 genes were informative in two of the three datasets. After the removal of the uninformative genes, 6025 genes were used in subsequent statistical analysis.

*Insert Figure 1 (Slide 1)*

**Figure 1. Venn diagram of informative genes in each dataset**

2. 314, 72 and 266 genes were identified as significantly changed in the ANIT, CPF and BEA datasets, respectively, in t-test. Of these genes, only 66 genes were significantly changed in at least two datasets, while the majority of gene expression changes appear to be chemical specific. A total of 584 independent genes were significantly changed in these three datasets.

*Insert Figure 2 (Slide 2)*

**Figure 2. Venn diagram of significant gene expression changes as identified by t-test after chemical treatments**

3. 92, 94 and 146 genes were identified as significantly changed in the ANIT, CPF and BEA datasets, respectively, in ANOVA. Of these genes, only 28 genes were significantly changed in at least two datasets. This is consistent with the results of t-test that the majority of gene expression changes are chemical specific. A total of 303 independent genes were significantly changed in these three datasets.

*Insert Figure 3 (Slide 3)*

**Figure 3. Venn diagram of significant gene expression changes as identified by ANOVA after chemical treatments**

4. A total of 441 genes were identified as significantly changed multi-class SAM analysis.
5. Comparison of the gene lists obtained different statistical analysis revealed that a total of 1075 independent genes were statistically significantly in the combined dataset. Of these 1075 genes, 213 genes were identified in at least two methods, while 40 genes were identified in all three methods.

*Insert Figure 4 (Slide 4)*

**Figure 4. Venn diagram of significant gene expression changes as identified by different statistical methods**

6. Classification of all samples using the expression profiles of these 1075, 213 and 40 genes were performed, and the result demonstrated the expression profiles of the 40 common genes provide the best discrimination regarding chemical treatments, as well as the doses of the treatment.

*Insert Figure 5 (Slide 5)*

**Figure 5. Principal Component Analysis of all samples using the expression profiles of all 1075 significantly changed as identified by different statistical methods**

*Insert Figure 6 (Slide 6)*

**Figure 6. Principal Component Analysis of all samples using the expression profiles of 213 genes, which were identified as significantly changed by at least two statistical methods**

*Insert Figure 7 (Slide 7)*

**Figure 7. Principal Component Analysis of all samples using the expression profiles of 40 genes, which were identified as significantly changed by all three statistical methods**

7. Examination of the functions of these 40 genes revealed that major biological processes are affected including cytoskeleton organization and biogenesis, ATP synthesis, carbohydrate metabolism, cell adhesion, protein biosynthesis, and stress response/xenobiotic metabolism.

**Table 1. Description of the 40 common genes identified as significantly changed using three different statistical methods**

*Insert Table*

## **CONCLUSION and DISCUSSION**

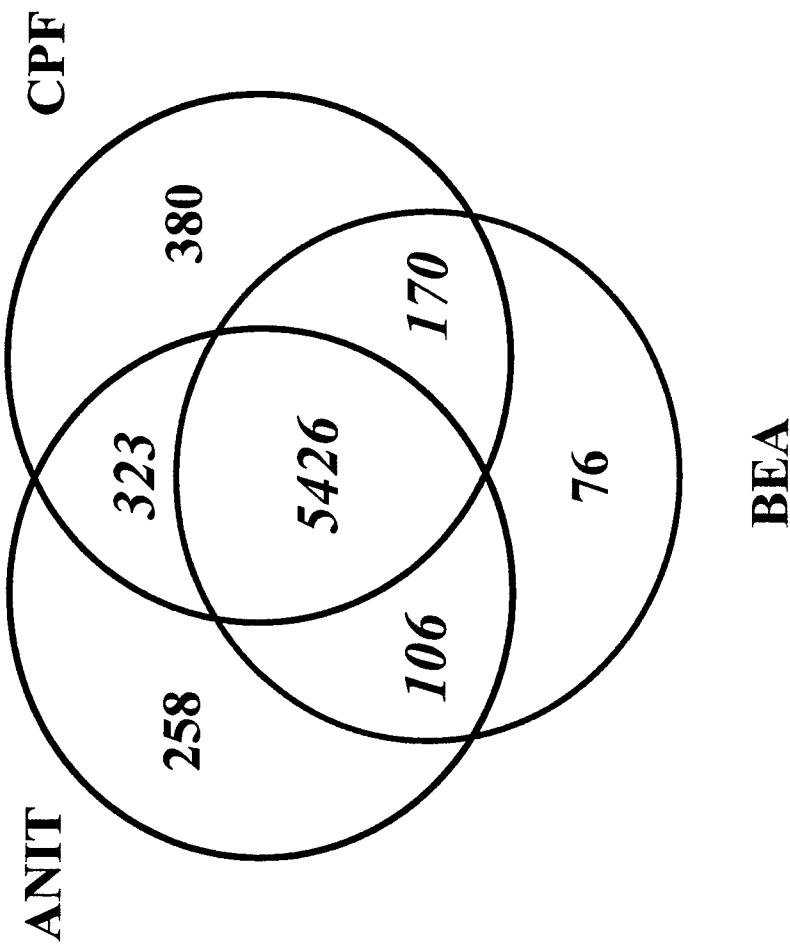
1. Exposure of rats to chemicals with different target organ toxicity results in gene expression changes in whole blood.
2. Different gene sets were identified as significantly changed using different statistical methods
3. Classification of samples grossly corresponding to the chemical and dose of the treatment was achieved using the gene expression profiles of the 40 genes that were identified as significantly changed using all three statistical methods.
4. Although common biological processes were affected by chemical treatments with different target organ toxicity, successful classification of the samples derived from different experiments suggested that the expression of these genes were altered in a chemical-dependent manner.
5. The result of this study suggests that whole blood gene expression changes may be used as surrogate biomarkers for exposure to chemicals, including those with different target organ toxicity.

## **REFERENCES**

1. Rodriguez-Garay EA. Cholestasis: human disease and experimental animal models. *Ann Hepatol.* 2003 Oct-Dec;2(4):150-8.
2. LeSage G, Glaser S, Alpini G. Regulation of cholangiocyte proliferation. *Liver.* 2001 Apr;21(2):73-80.
3. Roth RA, Dahm LJ. Neutrophil- and glutathione-mediated hepatotoxicity of alpha-naphthylisothiocyanate. *Drug Metab Rev.* 1997 Feb-May;29(1-2):153-65.
4. Lotti M, Moretto A. Organophosphate-induced delayed polyneuropathy. *Toxicol Rev.* 2005;24(1):37-49.

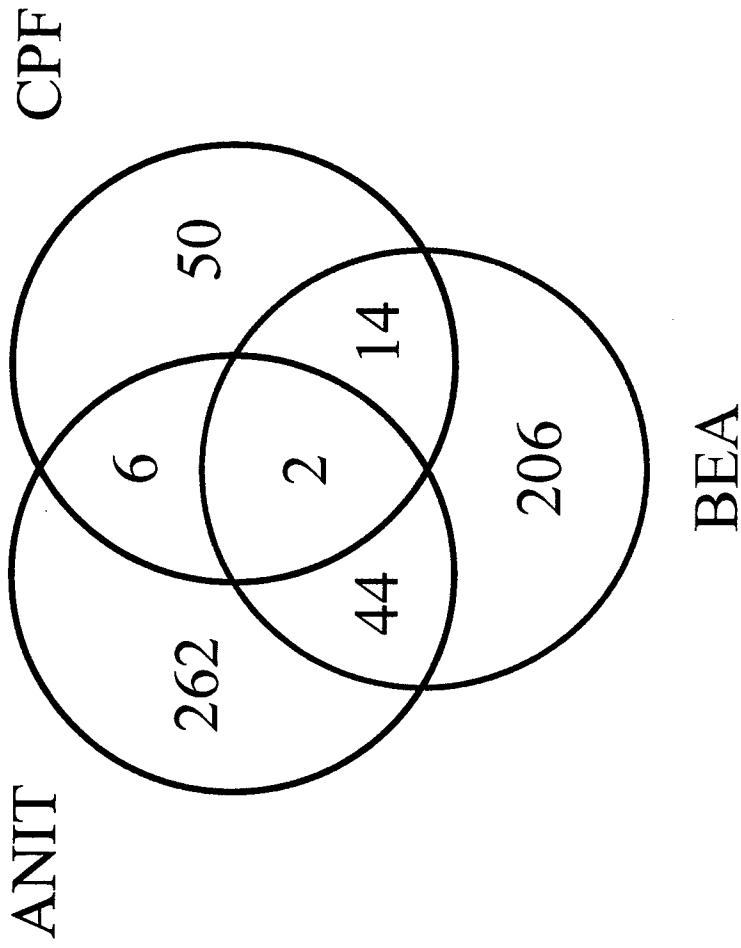
5. Galloway T, Handy R. Immunotoxicity of organophosphorous pesticides. *Ecotoxicology*. 2003 Feb-Aug;12(1-4):345-63.
6. Kinemuchi H, Sugimoto H, Obata T, Satoh N, Ueda S. Selective inhibitors of membrane-bound semicarbazide-sensitive amine oxidase (SSAO) activity in mammalian tissues. *Neurotoxicology*. 2004 Jan;25(1-2):325-35.
7. Anderson WP, Woods RL, Thomas CJ, Szenasi G, Evans RG. Renal medullary antihypertensive mechanisms. *Clin Exp Pharmacol Physiol*. 1995 Dec;22(12):S426-9.
8. Bretz F, Landgrebe J, Brunner E. Multiplicity issues in microarray experiments. *Methods Inf Med*. 2005;44(3):431-7.
9. Qian HR, Huang S. Comparison of false discovery rate methods in identifying genes with differential expression. *Genomics*. 2005 Oct;86(4):495-503.
10. Guo X, Pan W. Using weighted permutation scores to detect differential gene expression with microarray data. *J Bioinform Comput Biol*. 2005 Aug;3(4):989-1006.
11. Wu B. Differential gene expression detection using penalized linear regression models: the improved SAM statistics. *Bioinformatics*. 2005 Apr 15;21(8):1565-71.
12. Zhao Y, Pan W. Modified nonparametric approaches to detecting differentially expressed genes in replicated microarray experiments. *Bioinformatics*. 2003 Jun 12;19(9):1046-54.

## Informative Genes



**Filtered Dataset:  $5426 + 323 + 106 + 170 = 6025$  Genes**

## T-test

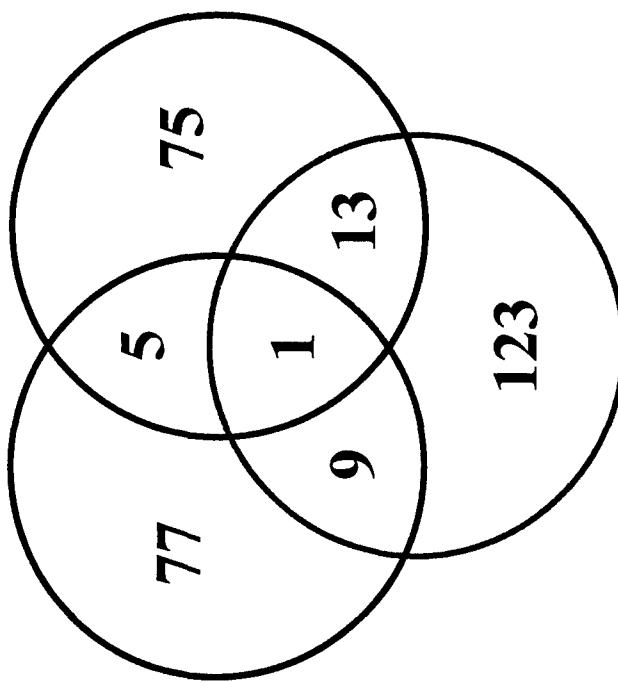


Total Gene Expression Changes: 584 Genes

## ANOVA

ANIT

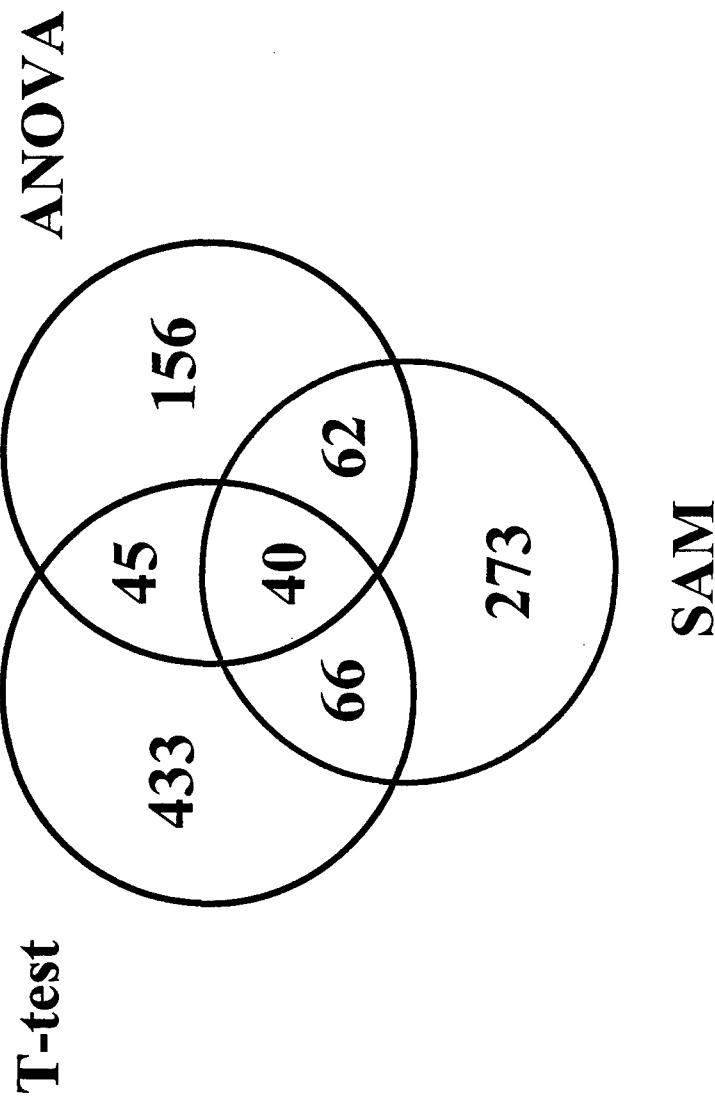
CPF



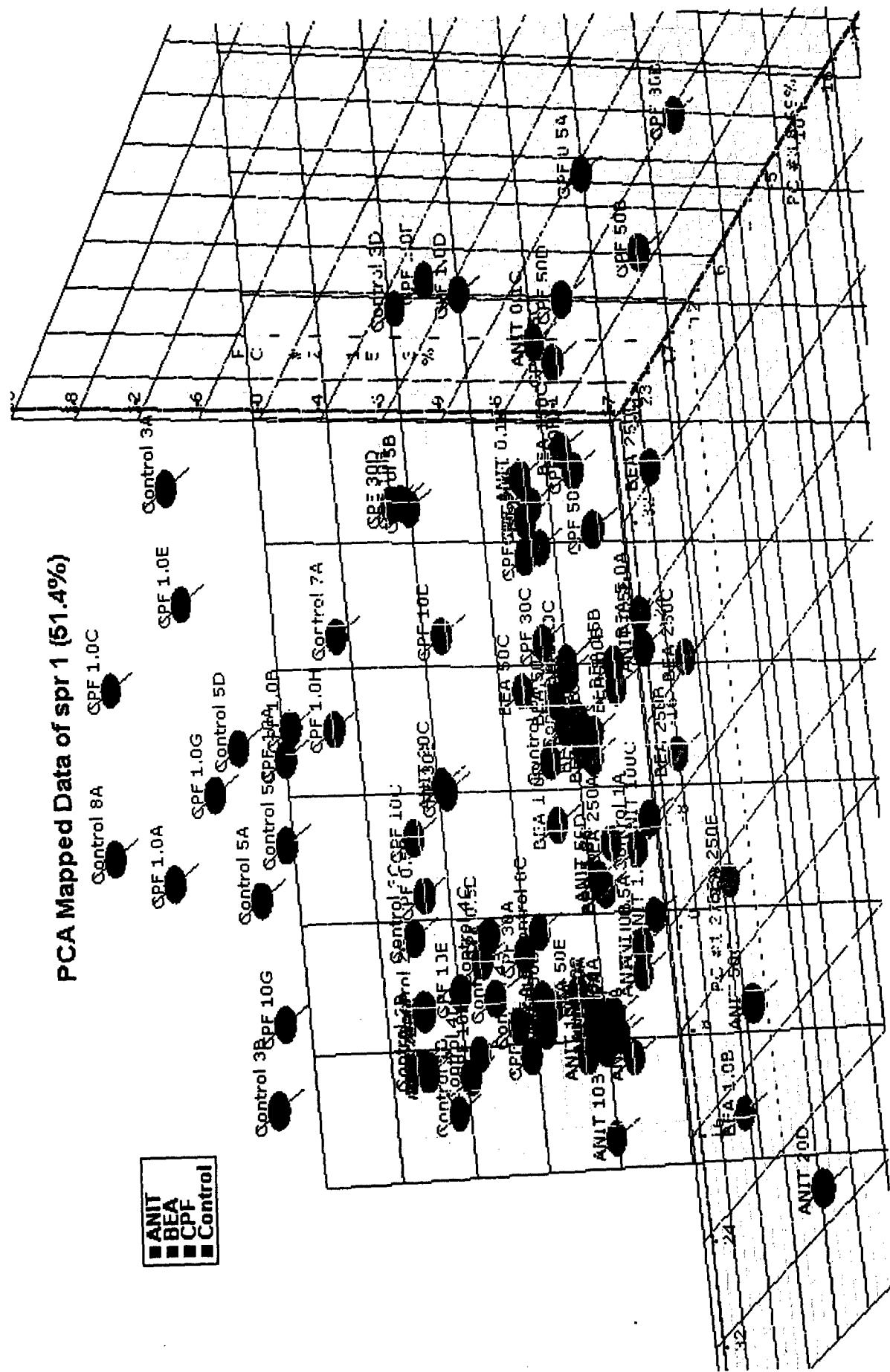
BEA

Total Gene Expression Changes: 303 Genes

## Combined Dataset

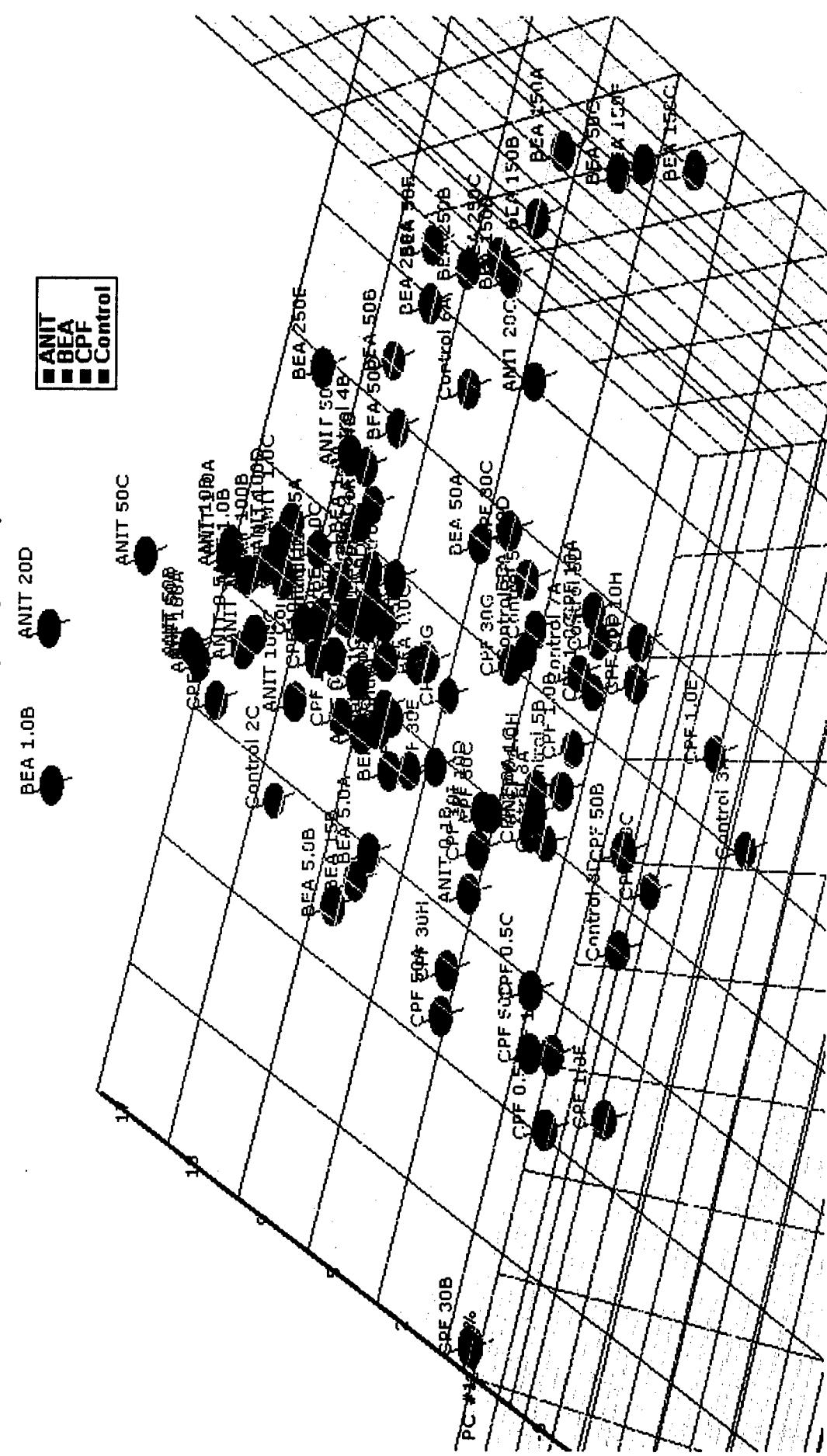


## Input Data: 1075 Genes



## Input Data: 213 Genes

PCA Mapped Data of spr 1 (55.1%)



## Input Data: 40 Genes

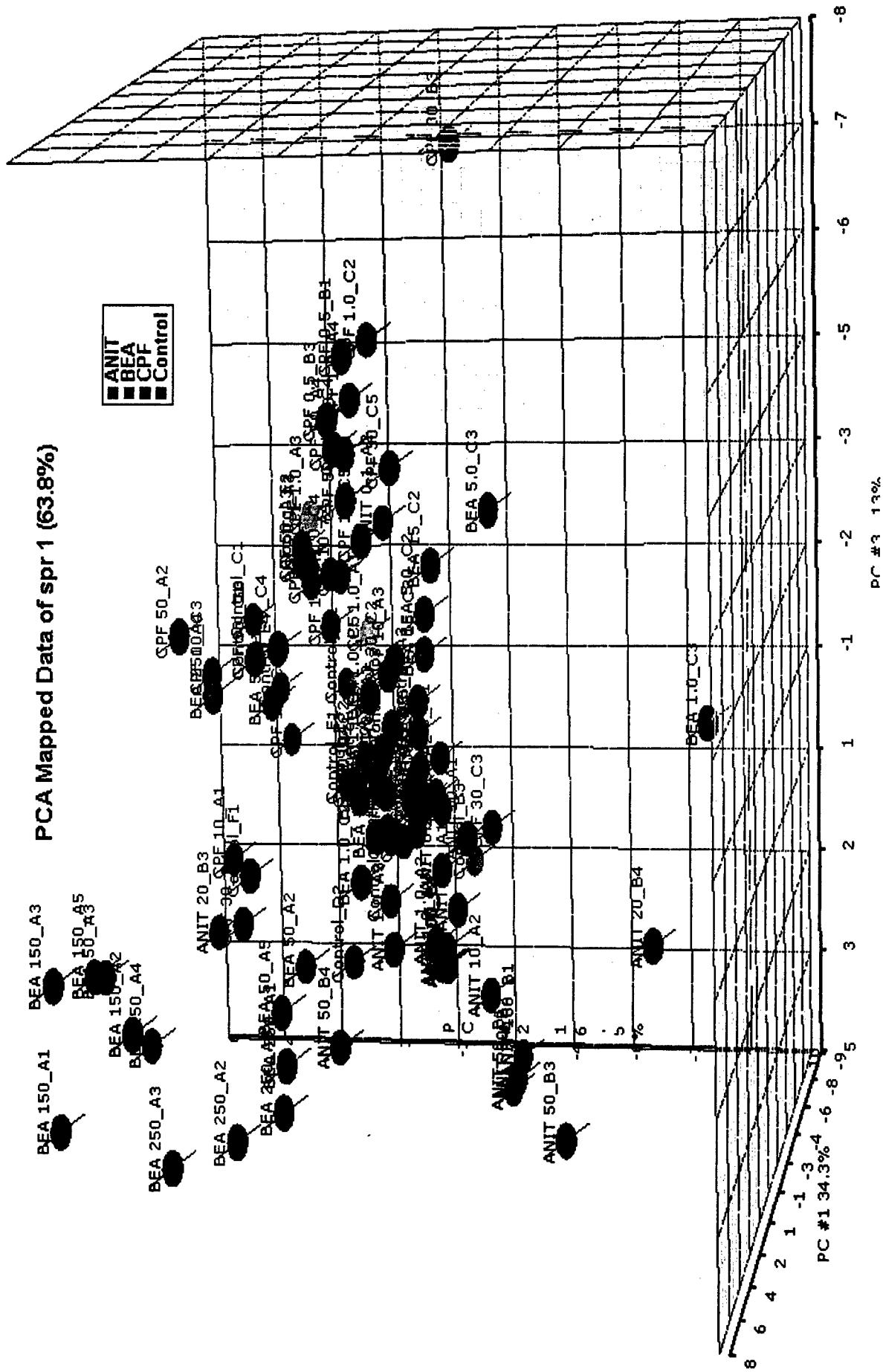


Table 1. Description of the 40 common genes identified as significantly changed using three different statistical methods

| Probe Set ID | Gene Title  | GO Biological Process Description   |
|--------------|---|---|
| 1372577_at   | actin related protein 2/3 complex, subunit 4 (predicted)                  | actin filament polymerization   |
| 1370184_at   | cofilin 1   | actin cytoskeleton organization and biogenesis, protein import into nucleus, Rho protein signal transduction      |
| 1398835_at   | actin, beta   | cell motility, cytoskeleton organization and biogenesis   |
| 1398836_s_at | actin, beta   | cell motility, cytoskeleton organization and biogenesis   |
| 1386996_at   | myosin light chain, regulatory B  | cytoskeleton organization and biogenesis, muscle development  |
| 1367605_at   | profilin 1  | cytoskeleton organization and biogenesis, actin polymerization and/or depolymerization                            |
| 1374388_at   | EF hand domain containing 2 (predicted)                                   | muscle development  |
| 1398755_at   | ATPase, H <sup>+</sup> transporting, V0 subunit C                         | ATP synthesis coupled proton transport, lysosomal lumen acidification   |
| 1367724_a_at | ATPase, H <sup>+</sup> transporting, V0 subunit E isoform 1               | ATP synthesis coupled proton transport  |
| 1371334_at   | integral membrane protein 2C (predicted)                                  | ATP binding   |
| 1367557_s_at | glyceraldehyde-3-phosphate dehydrogenase                                  | glucose metabolism, glycolysis, apoptosis   |
| 1367999_at   | aldehyde dehydrogenase 2  | carbohydrate metabolism, alcohol metabolism   |
| 1369931_at   | similar to pyruvate kinase (EC 2.7.1.40) isozyme M2                       | carbohydrate metabolism, alcohol metabolism   |
| 1367617_at   | aldolase A  | fructose metabolism, glycolysis   |
| 1370445_at   | phosphatidylserine-specific phospholipase A1                              | lipid metabolism  |
| 1367681_at   | CD151 antigen   | cell adhesion   |
| 1387952_a_at | CD44 antigen  | cell adhesion, defense response, ureteric bud branching   |
| 1388697_at   | inositol polyphosphate-5-phosphatase A (predicted)                        | cell communication, inositol phosphate dephosphorylation  |
| 1399072_at   | fibroblast growth factor (acidic) intracellular binding protein           | signal transduction   |
| 1371872_at   | Nucleosome assembly protein 1-like 1                                      | DNA replication, nucleosome assembly  |
| 1370493_a_at | leukocyte immunoglobulin-like receptor, subfamily B, member 3 (predicted) | immune response   |
| 1370636_at   | pro-platelet basic protein  | platelet formation, regulation of progression through cell cycle, glucose transport, defense response to bacteria |
| 1388454_at   | Ornithine decarboxylase antizyme 2  | polyamine biosynthesis  |
| 1370003_at   | eukaryotic translation elongation factor 2                                | protein biosynthesis, translational elongation  |
| 1372735_at   | eukaryotic translation initiation factor 3 subunit k (predicted)          | protein biosynthesis, translational initiation  |
| 1398315_at   | ribosomal protein L15   | protein biosynthesis  |
| 1371297_at   | ribosomal protein L7a (predicted)   | protein biosynthesis  |
| 1371423_at   | mitochondrial ribosomal protein L41 (predicted)                           | mitochondrial protein biosynthesis  |
| 1370319_at   | peptidylprolyl isomerase F (cyclophilin F)                                | protein folding   |
| 1367784_a_at | clusterin   | response to stress, apoptosis   |
| 1388122_at   | glutathione-S-transferase, pi 1   | xenobiotic metabolism   |
| 1374654_at   | beta-transducin repeat containing   | ubiquitin cycle   |
| 1389291_at   | coiled-coil-helix-coiled-coil-helix domain containing 3 (predicted)       | mitochondrial gene transcription  |
| 1399117_at   | similar to RIKEN cDNA 1110059G10 (predicted)                              |   |
| 1372249_at   | Transcribed locus   |   |
| 1372564_at   | Transcribed locus   |   |
| 1372989_at   | Transcribed locus   |   |
| 1380600_at   | Transcribed locus   |   |
| 1388653_at   | Transcribed locus   |   |
| 1389342_at   | Transcribed locus   |   |